Altered Kidney CYP2C and Cyclooxygenase-2 Levels Are Associated with Obesity-Related Albuminuria

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Abstract

DEY, APARAJITA, ROGER S. WILLIAMS, DAVID M. POLLOCK, DAVID W. STEPP, JOHN W. NEWMAN, BRUCE D. HAMMOCK, AND JOHN D. IMIG. Altered Kidney CYP2C and cyclooxygenase-2 levels are associated with obesity-related albuminuria. *Obes Res.* 2004;12: 1278–1289.

Objective: To determine cytochrome P450 (CYP450) and cyclooxygenase (COX) expression and metabolite regulation and renal damage in the early stages of obesity-related hypertension and diabetes.

Research Methods and Procedures: Obese and lean Zucker rats at 10 to 12 weeks of age were studied. Blood pressure was measured in the conscious state using radiotelemetry. Blood glucose levels and body weight were measured periodically. Protein expression of CYP450 and COX enzymes in the kidney cortex, renal microvessels, and glomeruli was studied. The levels of CYP450 and COX metabolites in urine were measured, and urinary albumin excretion, an indicator of kidney damage, was measured.

Results: Body weight and blood glucose averaged 432 ± 20 grams and 105 ± 5 mg/dl, respectively, in obese Zucker rats as compared with 320 ± 8 grams and 91 ± 5 mg/dl, respectively, in age-matched 10- to 12-week-old lean Zucker rats. Renal microvascular CYP4A and COX-2 protein levels were increased 2.3- and 17.0-fold, respectively, in obese Zucker rats. The protein expression of CYP2C11 and CYP2C23 was decreased 2.0-fold in renal microvessels isolated from obese Zucker rats when compared with lean Zucker rats. The urinary excretion rate of thromboxane B_2 was increased significantly in obese Zucker as compared with lean Zucker rats (22.0 ± 1.8 vs. 13.4 ± 1.0 ng/d). Urinary albumin excretion, an index of kidney damage, was increased in the obese Zucker rat at this early age.

Discussion: These results suggest that increased CYP4A and COX-2 protein levels and decreased CYP2C11 and CYP2C23 protein levels occur in association with microalbuminuria during the onset of obesity-related hypertension and type 2 diabetes.

Key words: obese Zucker rats, epoxyeicosatrienoic acids, prostaglandins, type 2 diabetes, nephropathy

Introduction

Obesity, a major nutritional disorder in the United States, leads to the development of a cluster of cardiovascular risk factors collectively known as Syndrome X. Syndrome X is a polygenic disorder that involves the clustering of metabolic and cardiovascular risk factors such as obesity, insulin resistance, type 2 diabetes, dyslipidemia, endothelial dysfunction, and hypertension (1,2). Obesity, along with diabetes, hypertension, hyperlipidemia, and other risk factors, contributes to the progression of kidney disease and to the development of diabetic nephropathy (3). Nephropathy occurs in 20% to 40% of diabetics and is the leading cause of end-stage renal disease (4). The obese Zucker rat is one of the few animal models with characteristics similar to Syndrome X, including extensive renal damage (3,5–8). Obese Zucker rats possess a mutant leptin receptor that explains their uncontrolled appetite, resultant obesity, and its associated characteristics such as insulin resistance, hypercholesterolemia, hypertriglyceridemia, and hypertension (3,9). The progression of renal injury in the obese Zucker rat has

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been evaluated, but the contributions of locally generated hormonal and paracrine factors have not been well defined.

Epoxygenase enzymes, such as CYP2C11 and CYP2C23, metabolize arachidonic acid to epoxyeicosatrienoic acids (EETs)[1] and are abundantly expressed in the kidney. EETs have anti-inflammatory vascular actions and dilate renal vessels (10–12). In contrast, CYP4A is primarily responsible for 20-hydroxyeicosatetraenoic acid (20-HETE) generation, and this hydroxylase metabolite is a potent vasoconstrictor (10,11). In addition, the soluble epoxide hydrolase (sEH) converts the EETs to the less active dihydroxyeicosatrienoic acids (DHETs) (13). Linoleic acid is abundant in the plasma and can be converted to epoxyoctadecenoic acids (EPOMEs) and dihydroxyoctadecenoic acids (DHOMEs) by cytochrome P450 (CYP450) and sEH enzymes. Nevertheless, very little is known about the renal and cardiovascular actions of these linoleic acid metabolites. To date, studies of CYP expression and catalytic activity in the obese Zucker rat have been limited primarily to the liver (14–16). One aspect of this study will evaluate the renal microvessel regulation of these enzymatic pathways in the obese Zucker rat.

Prostaglandins (PGs) and thromboxane (TX), formed by the enzymatic oxidation of arachidonic acid and catalyzed by cyclooxygenase (COX)-1 and COX-2, are other important metabolites implicated in the progression of renal disease in diabetes (17–19). COX regulation in the obese Zucker rat and its association with microalbuminuria, an indicator of renal damage, are unknown.

Therefore, the present study aimed to determine whether CYP2C11, CYP2C23, CYP4A, sEH, COX-1, and COX-2 expression and their metabolite regulation are associated with microalbuminuria in the early stages of obesity-related hypertension and diabetes.

Research Methods and Procedures

Animals

All experiments involving animals were carried out according to the guidelines of the Medical College of Georgia Institutional Animal Care and Use Committee. Male obese Zucker rats (Charles River Laboratories, Wilmington, MA) weighing ~400 to 450 grams and male lean Zucker rats weighing 300 to 350 grams were used for this study. The rats were housed in separate cages and maintained in a temperature- and light-controlled room. Throughout the experimental period, animals had access to standard chow and drinking water. Surgical procedures were performed with animals under pentobarbital anesthesia (40 mg/kg intraperitoneal) and sterile conditions. Animals were euthanized at 10 to 12 weeks of age for tissue harvesting.

Telemetry Blood Pressure Measurements

The blood pressure of each animal in the conscious state was measured using the radiotelemetry method. Mean arterial pressure was continuously measured for 24 hours, and an average blood pressure was obtained. Telemetry transmitters were implanted and data collected as previously described (20). The Biotelemetry Core at the Medical College of Georgia provided assistance with these studies.

Blood Glucose Measurements

Blood glucose levels were measured from the tail vein of the rats after overnight intake of food and after 3 to 4 hours of food deprivation using a commercially available kit (Roche Diagnostics, Indianapolis, IN).

Harvesting of Kidney Cortex

The rats were euthanized after collection of urine and their kidneys quickly removed and weighed. After separation of the cortical section from each kidney, 0.2 grams of the kidney samples was lysed in a buffer containing 50 mM Tris, 150 mM NaCl, 0.02% Na azide, 0.1% sodium dodecyl sulfate, 1.0% Igepal Ca-630, and 0.5% deoxycholate (pH 8.0) containing protease inhibitors (10 μg/mL aprotinin, 1 mM phenylmethsulfonyl fluoride, and 10 μg/mL leupeptin) and homogenized. After homogenizing these tissues, the samples were centrifuged at 3000 rpm for 15 minutes at 4 °C, and the supernatant containing the cytosolic and microsomal fractions was used for the study. Samples were stored at −80 °C until used.

Isolation of Renal Microvessels

Renal microvessels were isolated according to a method described previously (21). Animals were anesthetized, and a midline abdominal incision was made. The abdominal aorta below the renal arteries was cannulated and the superior mesenteric and aorta above the renal arteries tied off with ligatures. The kidneys were cleared of blood by perfusion of the isolated aortic segment with ice-cold physiological salt solution (PSS) (145 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose). After the kidneys were rinsed of blood, the perfusate was changed to a similar solution containing 1% Evans blue in PSS.

The kidneys were removed from the animal, and decapsulated, and the renal medullary tissue removed. Renal cortical tissue was pressed with a spatula on a 180-μm stainless sieve, and the retentate was rinsed several times with ice-cold PSS. The vascular tissue remaining was drawn through an 18-gauge needle four times to shear off attached glomeruli. Renal microvessels with some attached tubules

[1] Nonstandard abbreviations: EET, epoxyeicosatrienoic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; sEH, soluble epoxide hydrolase; DHET, dihydroxyeicosatrienoic acid; EPOME, epoxyoctadecenoic acid; DHOME, dihydroxyoctadecenoic acid; CYP450, cytochrome P450; PG, prostaglandin; TX, thromboxane; COX, cyclooxygenase; PSS, physiological salt solution; PBST, phosphate-buffered saline containing 0.3% Tween-20; SHR, spontaneously hypertensive rat; GFR, glomerular filtration rate; TXB_2, thromboxane B_2; PGF, prostaglandin F.

were gently agitated and incubated for 1 to 1.5 hours at 37 °C in 20 mL of a gassed (95% O_2/5% CO_2) PSS solution containing 0.2 mg/mL each dithiothreitol, collagenase type 2 (200 to 300 U/mg), soybean trypsin inhibitor type I-S (10,000 benzoyl-L-arginyl ethyl ester U/mg protein), and albumin. After collagenase digestion, the incubation media were discarded, and the vessels were suspended in 20 mL of ice-cold PSS and placed on a nylon membrane (100-μm mesh) in a vacuum filtration apparatus and rinsed several times with ice-cold PSS. Microvessels retained on the nylon membrane were collected under a stereomicroscope to ensure that they were free of tubular contamination. Renal microvessels (80 mg of tissue) from obese Zucker or lean Zucker rats were quickly frozen in liquid nitrogen and stored at −80 °C. Renal microvessels were homogenized in lysis buffer and stored as described for kidney cortex.

Isolation of Glomeruli

Glomeruli were isolated from rat kidneys by a modified procedure as described previously (22). The kidney cortex was dissected free and cut into small pieces with a surgical blade. The tissues were then poured onto a stainless steel 60-mesh screen (pore size: 200 μm), pressed with spatula, and rinsed with phosphate-buffered saline. Glomeruli-enriched tissue was retained on 200-mesh screen (pore size: 75 μm). The glomeruli were collected and lysed in radioimmunoprecipitation assay buffer containing 100 mM Tris (pH 7.4), 0.1 M EDTA, Triton X-100, 20% glycerol, 1 mM sodium deoxycholate, 5% sodium fluoride, and 100 mM sodium pyrophosphate, centrifuged, and the supernatant containing cytosolic and microsomal fraction was stored at −80 °C until further use.

CYP, sEH, and COX Protein Expression in Kidney

The protein expression of CYP4A, CYP2C11, CYP2C23, sEH, and COX in the kidney cortex, renal microvessels, and glomeruli was studied. The concentration of protein in these tissues was determined by the method of Lowry et al. (23). Kidney samples (cortex, renal microvessels, and glomeruli) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% Tris-glycine gel, and proteins were transferred electrophoretically to a 0.4-μm nitrocellulose membrane. Molecular weight markers (20 to 120 kDa) were used to determine the approximate molecular masses. The nonspecific binding sites were blocked by incubating the membranes at 4 °C in a blocking solution containing 20% nonfat dry milk in phosphate-buffered saline containing 0.3% Tween-20 (PBST). The membranes were washed with PBST and incubated with the primary antibody for 2 hours at room temperature. The primary antibodies that were used for CYP4A, CYP2C11, CYP2C23, sEH, and COX were goat antirat CYP4A (1:2000; BD Gentest, Woburn, MA), goat antirat CYP2C11 (1:500, Gentest), rabbit antirat CYP2C23 (1:5000; Dr. Capdevila, Nashville, TN), rabbit antimouse sEH (1:2000; Dr. Hammock, Davis, CA), goat antimouse COX-1 (1:100; Santa Cruz Biochemicals, Santa Cruz, CA), and rabbit antimouse COX-2 (1:1000; Cayman Chemical, Ann Arbor, MI), respectively. The blots were washed in PBST or 100 mM Tris-HCl (COX) and incubated in their respective secondary antibodies for 1 hour. The secondary antibodies for CYP4A, CYP2C11, CYP2C23, sEH, COX-1, and COX-2 were donkey antigoat IgG-HRP (1:40,000), rabbit antigoat IgG-HRP (1:30,000), goat antirabbit IgG-HRP (1:100,000), goat antirabbit IgG-HRP (1:40,000), rabbit antigoat IgG-HRP (1:40,000), and goat antirabbit IgG-HRP (1:20,000), respectively. After incubation with the secondary antibodies, the membranes were washed with PBST for a suitable period of time, and the band detection was done using enhanced chemiluminescence Western blotting. Band intensity was measured densitometrically, and the values were factored for β-actin.

Measurement of Urinary CYP450-Dependent Arachidonic Acid Metabolites

The urinary levels of arachidonic acid metabolites from obese and lean rats were measured as described previously (24). Animals were housed in separate metabolic cages that efficiently separated urine from food and feces for the entire experimental period. Urine was collected in a conical tube containing 5 mg of triphenylphosphine and cooled by dry ice. Samples were stored at -80°C until assayed. Sample aliquots (4 mL) were removed from thawed samples after mixing. These subaliquots were then spiked with analytical surrogates and extracted twice with 2 mL of ethyl acetate. The combined organic extracts were dried under nitrogen and redissolved in 100 μL of methanol and spiked with internal standards. An aliquot (10 μL) of the methanolic extract was then separated by reverse-phase high-performance liquid chromatography and analyzed by negative mode electrospray ionization and tandem mass spectroscopy as previously described (24).

Measurement of Urinary 6-Keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, TXB_2, and Albumin

The levels of 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2 (prostaglandin F), and thromboxane B_2 (TxB_2) in urine were measured using enzyme immunoassays according to the manufacturer's instructions (Cayman Chemical). The urinary albumin level was measured by using a competitive enzyme-linked immunosorbent assay (Nephrat; Exocell, Inc., Philadelphia, PA).

Statistical Analysis

All data are presented as mean ± SE. Student's unpaired two-tailed test was employed to calculate the statistical significance between obese Zucker and lean Zucker rats. $p < 0.05$ was considered to be significant when compared with the controls.

Table 1. Physiological parameters in 10- to 12-week-old obese and lean Zucker rats

	Lean ($n = 12$)	Obese ($n = 12$)
Blood glucose (mg/dL)	90.5 ± 4.7	105.2 ± 4.8*
Blood pressure (mm Hg)	94.0 ± 4.0	103.0 ± 6.0
Body weight (grams)	319.0 ± 7.7	432.3 ± 19.8*
Urinary volume (mL/d)	11.3 ± 0.7	19.6 ± 2.2*
Urinary Na^+ excretion rate (mmol/d)	1.3 ± 0.1	2.0 ± 0.2*
Urinary K^+ excretion rate (mmol/d)	4.5 ± 0.2	7.2 ± 0.7*
Urinary Cl^- excretion rate (mmol/d)	2.2 ± 0.1	3.4 ± 0.3*
Albumin (mg/d)	0.4 ± 0.2	7.0 ± 3.1*

Values are mean ± SE.
* Significant difference when compared with lean Zucker rats. $p < 0.05$.

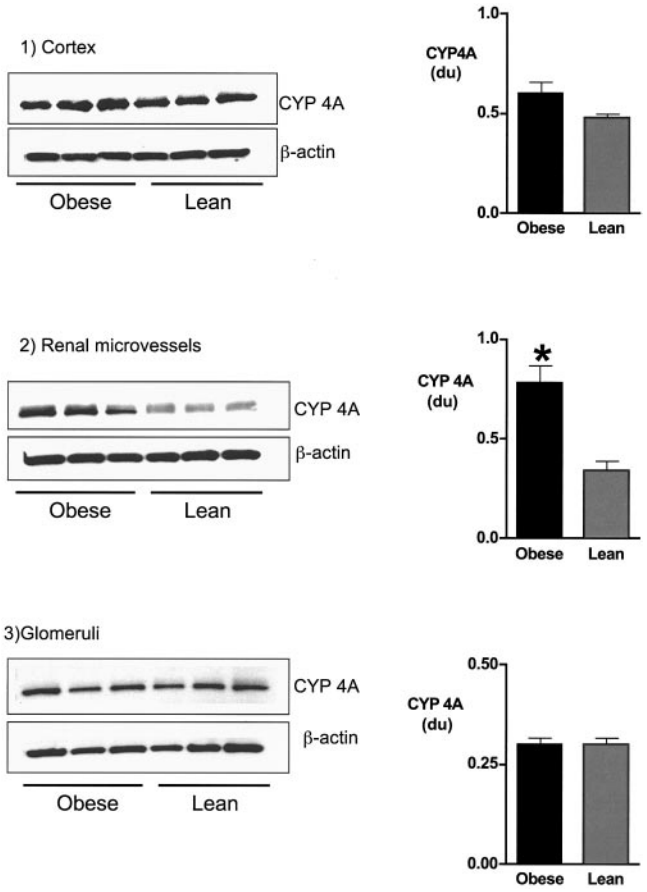

Figure 1: (Left) Renal cortical (1), microvascular (2), and glomerular (3) CYP4A protein expression: representative Western blots showing 52-kDa CYP4A protein bands in 10- to 12-week-old obese (lanes 1 to 3) and 10- to 12-week-old lean (lanes 4 to 6) Zucker rats. (Right) Densitometric values (du) for renal cortical, microvascular, and glomerular levels (10 μg/lane; $n = 6$/group) normalized to expression of β-actin. Values are mean ± SE. * Significant difference vs. lean Zucker rats.

Results

Physiological Parameters in Zucker Rats

The obese Zucker rats weighed 432 ± 20 grams, and their average blood glucose was 105 ± 5 mg/dl as compared with age-matched lean Zucker rats having body weight of 320 ± 8 grams and blood glucose of 91 ± 5 mg/dl (Table 1). Urinary excretion volume was 74% greater in obese Zucker rats (19.6 ± 2.2 mL/d) than in lean Zucker rats (11.3 ± 0.7 mL/d). The obese Zucker rats had higher Na^+, K^+, and Cl^- urinary excretion rates than the lean animals, and microalbuminuria was present (7.0 ± 3.1 mg/d) in 10- to 12-week-old obese Zucker rats, whereas the lean rats excreted little or no albumin in their urine (0.4 ± 0.03 mg/d).

CYP450 Protein Expression in Kidney Cortex, Renal Microvessels, and Glomeruli of Zucker Rats

Figure 1 depicts the results of Western-blot and densitometric analysis of CYP4A protein expression in Zucker rats. CYP4A expression was significantly increased in the renal microvessels of obese Zucker rats (2.5 fold) when compared with age-matched lean Zucker rats. CYP4A expression was also slightly increased in cortical tissues of obese Zucker rats; however, the increase in CYP4A in the cortex did not reach statistical significance. In contrast, the glomerular CYP4A protein expression was not different in obese and lean Zucker rats.

As evident from Figure 2, Western blots and densitometric analysis revealed that the CYP2C23 protein expression was not different in the kidney cortex of 10- to 12-week-old obese Zucker and lean Zucker rats. A similar pattern of protein expression was also observed in the glomeruli of these animals. In contrast, CYP2C23 protein expression was decreased 2.1-fold in the renal microvessels isolated from obese Zucker rats when compared with lean Zucker rats.

Figure 3 illustrates the results of Western-blot and densitometric analysis of CYP2C11 protein expression in different tissues of Zucker rats. There was no difference observed in the expression of CYP2C11 in kidney cortex of obese Zucker and lean Zucker rats. The protein expression of CYP2C11 was decreased 2.0-fold in the microvessels isolated from obese Zucker rats when compared with lean Zucker rats. Interestingly, a decrease in CYP2C11 expression was also observed in glomerular tissue isolated from obese Zucker rats.

As evident from Figure 4, Western blots and densitometric analyses revealed that cortical, renal microvascular, and

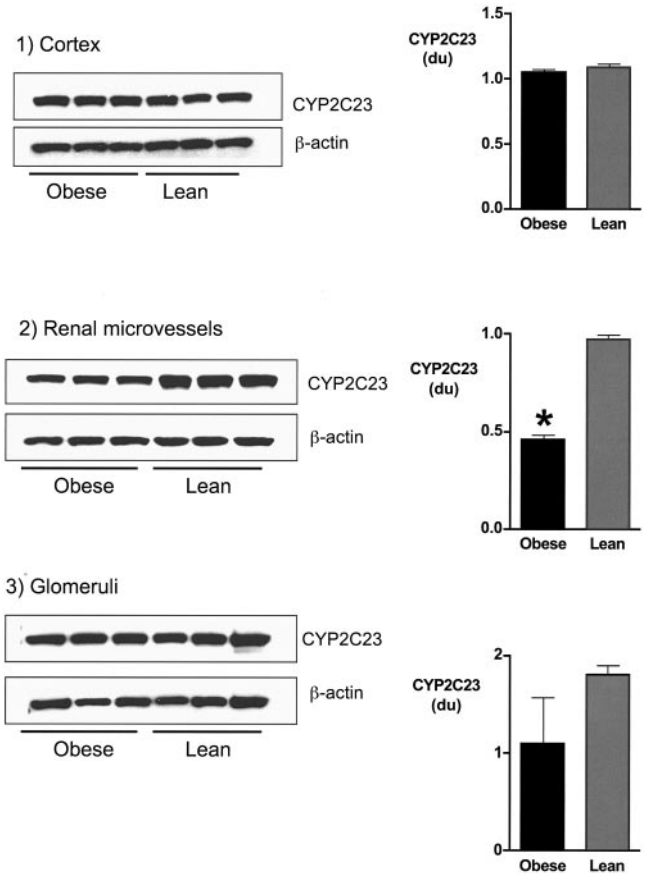

Figure 2: (Left) Renal cortical (1), microvascular (2), and glomerular (3) CYP2C23 protein expression: representative Western blots showing 48-kDa CYP2C23 protein bands in 10- to 12-week-old obese (lanes 1 to 3) and 10- to 12-week-old lean (lanes 4 to 6) Zucker rats. (Right) Densitometric values (du) for renal cortical, microvascular, and glomerular levels (10 μg/lane; n = 6/group) normalized to expression of β-actin. Values are mean ± SE. * Significant difference vs. lean Zucker rats.

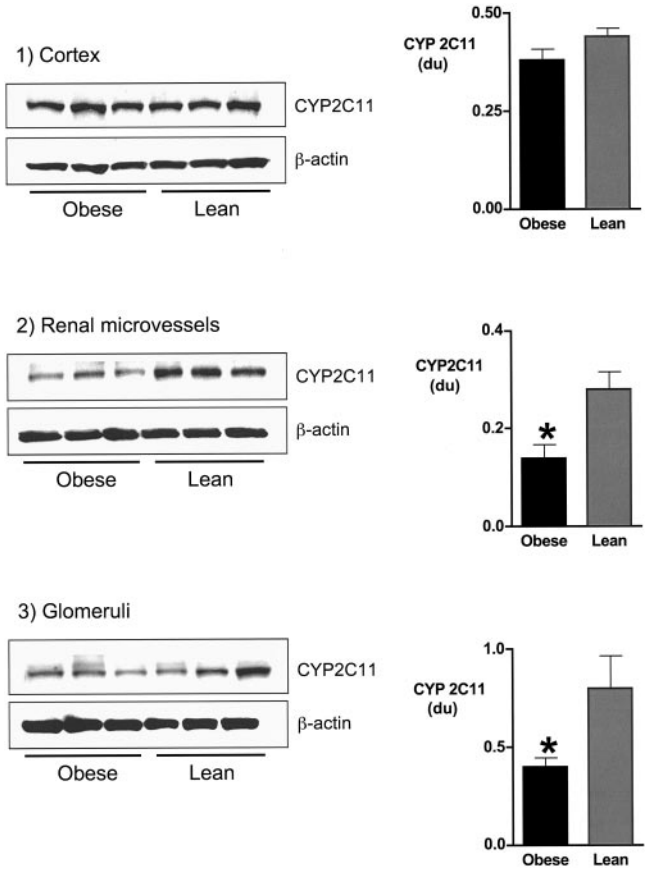

Figure 3: (Left) Renal cortical (1), microvascular (2), and glomerular (3) CYP2C11 protein expression: representative Western blots showing 48-kDa CYP 2C11 protein bands in 10- to 12-week-old obese (lanes 1 to 3) and 10- to 12-week-old lean (lanes 4 to 6) Zucker rats. (Right) Densitometric values (du) for renal cortical, microvascular, and glomerular levels (10 μg/lane; n = 6/group) normalized to expression of β-actin. Values are mean ± SE. * Significant difference vs. lean Zucker rats.

glomerular sEH expression were not different in the two groups of animals (Figure 4). Obese Zucker and lean Zucker rat kidney tissues had similar levels of sEH expression.

COX Protein Expression in Kidney Cortex, Renal Microvessels, and Glomeruli of Zucker Rats

Figure 5 shows the Western blots of COX-1 in kidney cortex, renal microvessels, and glomeruli of obese and lean Zucker rats. Although a significant increase (1.5-fold) in renal microvascular COX-1 expression was observed in these animals, the increase in microvascular COX-1 was unaccompanied by an increase in COX-1 expression in cortex and glomeruli.

A significant increase in COX-2 expression was observed in the cortical and renal microvascular tissues of obese Zucker rats (Figure 6). COX-2 expression was increased 2.0- and 17.0-fold, respectively, in kidney cortex and microvessels of obese Zucker compared with lean Zucker rats. Microvascular COX-2 expression was characterized by a barely detectable band in lean Zucker rats. Also, there was no change in the expression of COX-2 in the glomerular tissues of these animals.

Urinary Excretion of COX and CYP450 Metabolites in Zucker Rats

Urinary prostanoid excretory levels determined by enzyme-linked immunosorbent assay are presented in Figure 7. The urinary TXB_2 excretion was 64% greater in obese Zucker rats than lean Zucker rats. Also, the levels of PGE_2 excreted in urine for a period of 24 hours were decreased 80% in obese Zucker rats. Urinary levels of 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ were unchanged in obese Zucker rats.

Urinary CYP450 arachidonic acid and linoleic acid metabolite excretion rates are presented in Table 2. Interest-

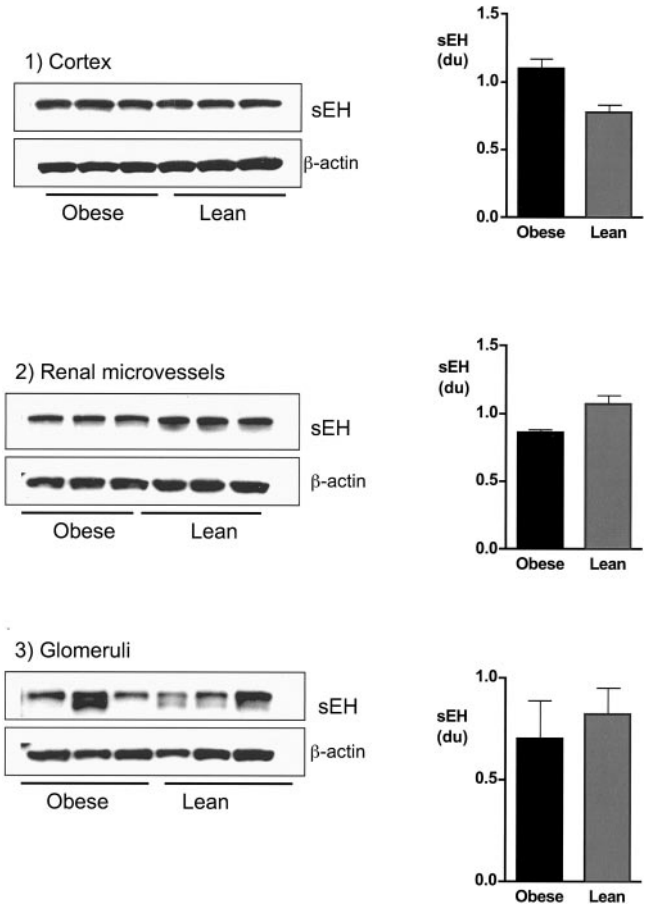

Figure 4: (Left) Renal cortical (1), microvascular (2), and glomerular (3) sEH protein expression: representative Western blots showing 62-kDa sEH protein bands in 10- to 12-week-old obese (lanes 1 to 3) and 10- to 12-week-old lean (lanes 4 to 6) Zucker rats. (Right) Densitometric values (du) for renal cortical, microvascular, and glomerular levels (10 μg/lane; n = 6/group) normalized to expression of β-actin. Values are mean ± SE. * Significant difference vs. lean Zucker rats.

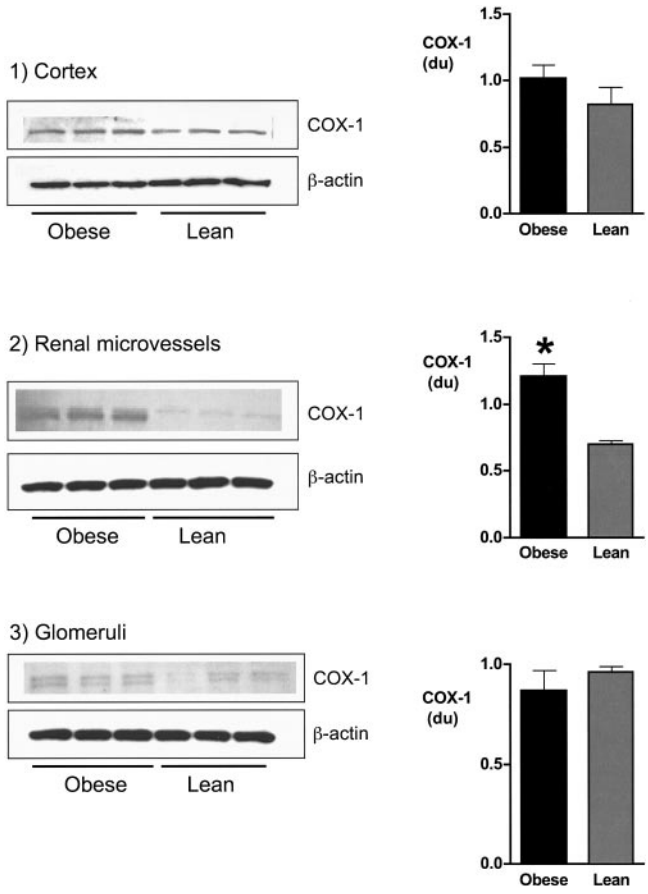

Figure 5: (Left) Renal cortical (1), microvascular (2), and glomerular (3) COX-1 protein expression: representative Western blots showing 68-kDa COX-1 protein bands in 10- to 12-week-old obese (lanes 1 to 3) and 10- to 12-week-old lean (lanes 4 to 6) Zucker rats. (Right) Densitometric values (du) for renal cortical, microvascular, and glomerular levels (10 μg/lane; n = 6/group) normalized to expression of β-actin. Values are mean ± SE. * Significant difference vs. lean Zucker rats.

ingly, we observed increased EPOMEs in the urine of the obese Zucker rats when compared with age-matched lean Zucker rats. A similar trend was observed with the EETs, but this did not reach statistical significance. There was no difference in the DHOME or DHET urinary levels between the two groups. The composition of the linoleic acid DHOMEs and EPOMEs was similar in the obese and lean Zucker rats, with ~80% of the EPOME as 12,13-EPOME and 76% of the DHOME as 12,13-DHOME. As for CYP450 arachidonic acid metabolites, the distributions of both EETs and DHETs in the obese and lean Zucker rats were equivalent and similar to previously reported profiles in rat urine (24). Although the 14,15-EET was the major EET accounting for ~70% of the total EETs, the 5,6-DHET accounted for the majority of the DHETs in the Zucker rats. It should be noted that although the 5,6-EET is not accurately quantified by the applied procedure, the 5,6-DHET values are not elevated due to abiotic hydrolysis during sample analysis (24). In addition, although urinary 20-HETE was not detected, this compound can be actively metabolized to the corresponding 20-carboxy analog, as well as vasoconstrictive hydroxy-PGs that were not measured during this study (25).

Discussion

Obesity leads to a host of other metabolic disorders such as hypertension, diabetes, atherosclerosis, and chronic renal disease, many of which are interdependent (1,2). Abnormal functioning of the kidneys results in increased blood pressure in obese individuals and hypertension that, in turn, contributes to chronic renal disease (1). Among the major risk factors that contribute to the development and progres-

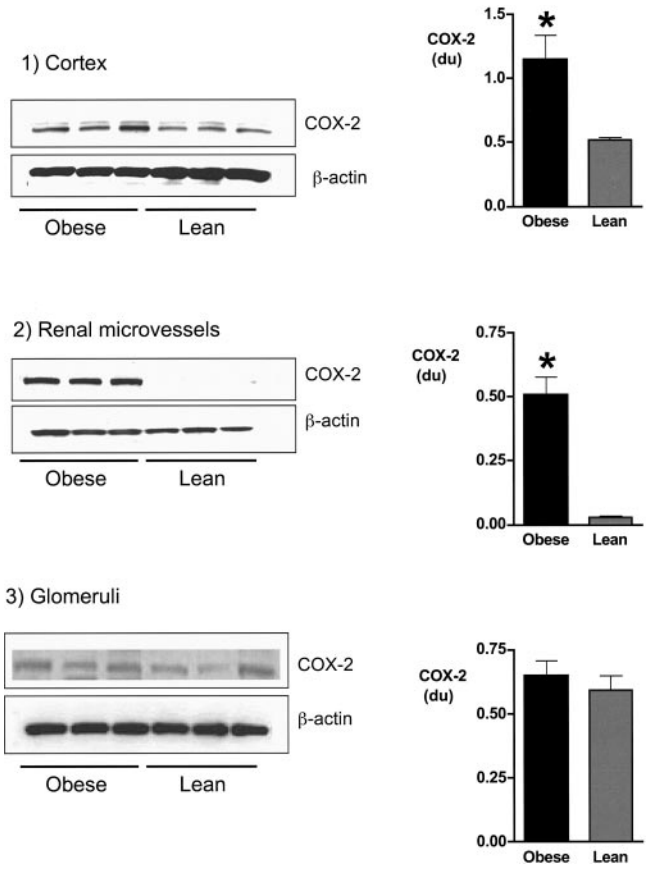

Figure 6: (Left) Renal cortical (1), microvascular (2), and glomerular (3) COX-2 protein expression: representative Western blots showing 72-kDa COX-2 protein bands in 10- to 12-week-old obese (lanes 1 to 3) and 10- to 12-week-old lean (lanes 4 to 6) Zucker rats. (Right) Densitometric values (du) for renal cortical, microvascular, and glomerular levels (10 μg/lane; n = 6/group) normalized to expression of β-actin. Values are mean ± SE. * Significant difference vs. lean Zucker rats.

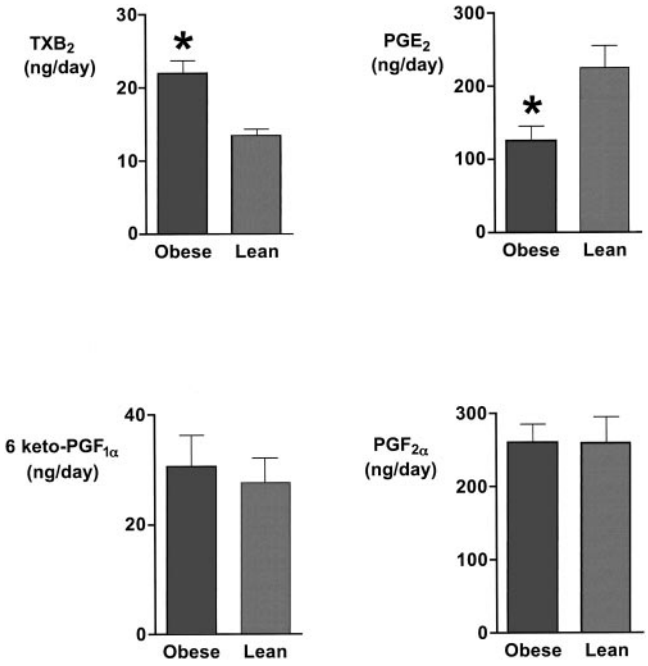

Figure 7: Urinary excretion rates of PGE_2, TxB_2, 6 keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$ in 10- to 12-week-old Zucker rats (n = 12/group). Values are mean ± SE. * Significant difference vs. lean Zucker rats.

sion of renal damage in diabetes are hyperglycemia, obesity, elevated blood pressure, and early hyperfiltration (2). Arterial blood pressure has a complex relationship with diabetic nephropathy, wherein nephropathy raises blood pressure and blood pressure accelerates the damage caused by nephropathy (26). Most type 2 diabetics are hypertensive (almost 80%); therefore, hypertension is an important risk factor that can be modified by numerous other factors that lead to the development and progression of renal damage in diabetes (26,27). The obese Zucker rat is a unique animal model because this rat strain provides an opportunity to study the complex relationships among obesity, hypertension, diabetes, and renal damage. The objective of the present study was to evaluate renal CYP450 and COX regulation in 10- to 12-week-old obese and lean Zucker rats.

The present study demonstrates that there is a small increase in blood glucose with increasing body weight in 10- to 12-week-old obese Zucker rats. In addition, the obese Zucker rat had a slightly elevated blood pressure. Although statistically significant elevations in blood pressure and blood glucose were observed, these values are well within the clinically normal range. Thus, the obese Zucker rat at 10 to 12 weeks of age is at a prehyperglycemic and prehypertensive stage. Increased urine volume and electrolyte excretion were evident in the obese Zucker rat, and this is consistent with increased food and water intake. Elevated urinary albumin was also observed in the 10- to 12-week-old obese Zucker rats as previously reported (3,5,8). Microalbuminuria is an early indicator of diabetic nephropathy and strongly predicts cardiovascular morbidity and mortality in diabetic patients (4,26–28). Early renal damage combined with an increase in systemic blood pressure ultimately is known to result in extracellular matrix accumulation, increased glomerular permeability, proteinuria, and glomerulosclerosis (4,26,27). Thus, we provide evidence that the progression of renal damage starts at a very early prehyperglycemic and prehypertensive stage in the obese Zucker rats.

We evaluated the regulation of kidney CYP450 enzymes to determine changes that occur in the 10- to 12-week-old obese Zucker rat. Consistent with an earlier report documenting hepatic CYP450 expression (15), this study shows an up-regulation of CYP4A expression in renal microvessels but not glomeruli of obese Zucker rats in the prehyper-

Table 2. Urinary CYP450 metabolites in 10- to 12-week-old obese and lean Zucker rats

Urinary oxylipid metabolites	Lean ($n = 11$)	Obese ($n = 10$)
EPOMEs (pmol/dL)	1708 ± 547	5623 ± 1779*
DHOMEs (pmol/d)	8014 ± 2347	7610 ± 1631
EETs (pmol/d)	10.8 ± 3.2	21.2 ± 5.3
DHETs (pmol/d)	155.2 ± 46.1	186.9 ± 45.7
12,13-EPOME	79 ± 2%	84 ± 1%*
9,10-EPOME	21 ± 2%	16 ± 1%
12,13-DHOME	76 ± 1%	76 ± 2%
9,10, DHOME	24 ± 1%	24 ± 2%
14,15-EET	64 ± 13%	71 ± 13%
11,12-EET	30 ± 13%	17 ± 13%
8,9-EET	ND	ND
5,6-EET†	6 ± 5%	12 ± 8%
14,15-DHET	23 ± 1%	14 ± 1%
11,12-DHET	5 ± 1%	2 ± 1%
8,9-DHET	2 ± 1%	1 ± 1%
5,6-DHET	70 ± 1%	83 ± 2%

Values are mean ± SE.

* Significant difference when compared with lean Zucker rats. $p < 0.05$.

† 5,6-EET recoveries with the implemented procedure are roughly 25%. Loss of this compound was apparently due to internal cyclization to the 5,6-delta lactone not hydrolysis to the 5,6-DHET (29). % indicates the percentage of specific regioisomeric EPOMEs, DHOMEs, EETs, and DHETs in total EPOMEs, DHOMEs, EETs, and DHETs, respectively.

glycemic stage (10 to 12 weeks). CYP4A enzymes are also involved in ω-hydroxylation of fatty acids, which is a major pathway for fatty acid oxidation in states of altered nutrient metabolism like diabetes, ketosis, and starvation (15). Leptin deficiency or the obese diabetic state may be responsible for the induction of CYP4A in the obese Zucker rat. CYP4A enzymes are primarily involved in the formation of 20-HETE, a potent vasoconstrictor of the preglomerular arterioles (11,21). In a study involving Sprague Dawley rats, daily intravenous injections of a 20-mer antisense CYP4A1 oligonucleotide for 5 days reduced the expression of vascular CYP4A1 and CYP4A2 proteins and the production of 20-HETE in renal arterioles accompanied by a modest decrease in arterial blood pressure (29). There is now overwhelming evidence that the increased production of 20-HETE, formed primarily through the catalytic activity of CYP4A1, results in increased vasoconstriction and hypertension (30). Studies of differential gene expression have identified CYP4A2 as upregulated in the spontaneously hypertensive rat (SHR), and production of 20-HETE is elevated in SHR during the development of hypertension (31). In agreement with our findings, Cyp4a10 and Cyp4a14 liver mRNA expression were also found to be elevated in obese mice (15). This homozygous C57BL/6J *ob/ob* mouse has a deficiency in leptin synthesis and develops phenotypic changes similar to the obese Zucker rat (15). In a study involving streptozotocin-induced diabetic rats, elevated CYP4A2 expression and increased ω-hydroxylation of arachidonic acid were observed in renal microsomes (32,33). Accordingly, the increase in renal microvascular CYP4A expression at the prehyperglycemic stage in the obese Zucker rat could contribute to the hypertension and consequent renal damage observed in these animals.

In contrast to the hydroxylase enzymes, the epoxygenase enzymes CYP2C11 and CYP2C23 were decreased in the renal microvessels of obese Zucker rats. We have also observed decreased renal CYP2C23 expression and activity in rats fed a high-fat diet (34). CYP2C enzymes are the major EET-forming enzymes in kidneys, and EET production can be altered by changes in the regulation of the expression and/or activity of these enzymes (11,35). EETs are endogenous constituents of human and rodent kidneys, and EET biosynthesis occurs throughout the nephron (35). EETs also augment sodium excretion and decrease cortical renin release (11). More importantly, EETs are endothelium-derived hyperpolarizing factors that are important mediators of vascular relaxation, most specifically in resistance-sized arteries where they regulate tissue blood flow (11). It has become apparent that epoxygenase metabolites also possess profibrinolytic effects and anti-inflammatory actions, and inhibit vascular smooth muscle proliferation (12,36,37). Therefore, a decrease in CYP2C enzymes could decrease the renal and cardiovascular protective actions of these metabolites.

Recent reports have suggested a significant role for sEH in the long-term regulation of arterial blood pressure (13,38). EETs are hydrated to their corresponding DHETs by sEH, and these DHETs lack the renal vasodilatory effects of their progenitor EETs (38). In SHR, an increase in the renal metabolism of EETs to DHETs occurs during the development of hypertension, indicating increased expression or activity of sEH (39). In male mice, deletion of the sEH gene tends to lower the blood pressure in these animals (40). EPOMEs can also be converted to DHOMEs by the sEH enzyme. Although there is limited information about the biological actions of linoleic acid metabolites, EPOMEs infused into conscious normotensive rats have demonstrated a slight decline in blood pressure but no change in heart rate (41). The renal and cardiovascular actions of the linoleic acid metabolites and their contribution of sEH to regulate these metabolites await further exploration. In any case, the current study failed to find any changes in sEH levels in the renal tissues isolated from obese Zucker rats.

The changes in excretion rates of urinary CYP450 linoleic acid and arachidonic acid metabolites in the obese Zucker rats do not correlate with changes in renal microvascular CYP450 enzyme expression. Although urinary excretions of the EPOMEs were elevated in the obese Zucker rats, the data were quite variable, and other concentration-dependent discriminations were not observed. However, the composition profiles of urinary oxylipids were similar to those previously reported for the SHR and Sprague-Dawley rats (24). As mentioned above, the renal vascular epoxygenase enzymes were decreased. These apparent contradictory findings could be due to the fact that urinary excretion of CYP450 metabolites does not directly reflect alterations in renal microvascular enzymes but may reflect renal tubular production. It is interesting to note that kidney cortex epoxygenase enzymes inspected here were not different between the obese and lean Zucker rats. Secondly, the obese Zucker rat is hyperphagic and would ingest more fatty acids and increase substrate available for the epoxygenases systemically, which could result in higher circulating concentrations and ultimately greater levels of renal clearance. Lastly, changes in phospholipid membrane composition have been observed in the tissues of obese Zucker rats (42,43). This change in phospholipid membrane composition has been linked to alterations in COX metabolism by the heart (42).

Next, we evaluated COX enzymes that are expressed at high levels in the kidney (44). COX-1 is typically expressed constitutively and generates physiologically active PGs that regulate renal blood flow (18). COX-2 is constitutively expressed in the kidney and can also be induced to form PGs involved in inflammation (18,44). COX products of arachidonic acid metabolism account for the endothelial dysfunction that occurs in both types 1 and 2 diabetes by causing acute increases in vascular tone, increased blood pressure, and vascular and cardiac remodeling that contributes to the vascular and renal complications in diabetes (45). In the present study, the expression of COX-1 was not changed significantly in the renal cortex and glomeruli but was increased in the renal microvessels of obese Zucker rats. Likewise, COX-2 protein expression was significantly increased in the renal cortex and microvessels of the obese Zucker rats. Earlier observations using other models of diabetes have suggested that increased COX-2 may contribute to diabetic nephropathy (17,19). In a study involving the streptozotocin diabetic rats, a model of type 1 diabetes, administration of the COX-2 inhibitor SC58236 resulted in decreased COX-2 expression, reduced proteinuria, and a decreased mesangial sclerosis index (17). The possible beneficial effects of COX-2 inhibitors in the obese Zucker rat remain unknown.

PGs formed through the metabolic actions of COX-2 play a vital role in vascular inflammation, modulating afferent arteriolar vasoconstriction and stimulation of renin release (11,18). PGE_2 is a dilator PG that increases renal blood flow and glomerular filtration rate (GFR) under conditions associated with decreased actual or effective circulating volume (46). Although increased PG production contributes to the large elevation of renal plasma flow and GFR in streptozotocin-induced diabetes, obese Zucker rats have unaltered or slightly elevated GFR at this age that progressively declines between 12 and 28 weeks of age (7,47–51). Obese Zucker rats also exhibit an impaired pressure natriuretic response that is associated with decreased vasodilator capacity compared with lean controls at 8 weeks of age (50). In contrast to the actions of PGE_2, TXA_2 is a vasoconstrictor that induces the transcription of type IV collagen, laminin, and fibronectin mRNA in the glomerular matrix and down-regulates the synthesis of heparin sulfate proteoglycan (52). TX mimetics have been shown to stimulate mesangial cell matrix production by both transforming growth factor-β-dependent and -independent pathways (53). PGI_2 has been shown to counteract these deleterious TXA_2 effects associated with progressive glomerular damage (52). In diabetic patients, an excessive vasoconstrictive and proaggregatory TXA_2 renal synthesis, concomitant with a decrease in vasodilatory and antiaggregatory PGE_2, has been found to influence renal functions such as protein excretion in diabetes (54–56).

We observed increased urinary TXB_2 excretion and decreased urinary PGE_2 excretion in 10- to 12- week-old obese Zucker rats. The changes in urinary TXB_2 and PGE_2 excretion were not accompanied by alterations in the levels of 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$. The changes in urinary COX metabolites observed in the obese Zucker rats cannot be fully explained by alterations in renal COX protein expression. Increased COX-1 and COX-2 levels could contribute to the increased urinary TXB_2 (57–59). Diuretic-induced increases in renal COX-2 mRNA levels have been associated with elevated urinary TXB_2 excretion (57). COX-2 is constitutively expressed in macula densa and thick ascending limb of the loop of Henle cells, and increased COX-2 expression in these cells could account for the increase in urinary TXB_2 in the obese Zucker rats (44,46,57). In any case, increased PGE_2 production in response to inflammation and elevated renal COX-2 levels do not fit with the observation of decreased urinary PGE_2 levels in the obese Zucker rat. One possible explanation is that COX-2 could be decreased in the renal medulla; however, the current study focused on vascular and glomerular regulation of COX-2. In addition, the regulation of the synthetases responsible for the production of TXB_2 and PGE_2 in the obese Zucker rat is unknown and has not been extensively studied in diabetic nephropathy.

Intriguingly, in a study involving noninsulin-dependent diabetic patients, the urinary excretion ratio of 6-keto $PGF_{1\alpha}$ to TXB_2 was decreased, and treatment of the patients with ozagrel, a specific TX synthetase inhibitor, reduced urinary

TXB_2 excretion leading to improvement in the 6-keto $PGF_{1\alpha}$-to-TXB_2 ratio (54). Noninsulin-dependent diabetic patients treated with ozagrel had decreased urinary albumin excretion and a reduced platelet aggregation rate (60). These observations are supported by the results of another study involving patients with diabetes who excreted more TXB_2 and less PGE_2 in their urine (61). Urinary PGs and TXB_2 excretion are elevated early in the development of streptozotocin-induced diabetes (47–49). In a study using streptozotocin diabetic rats at a later stage, the urinary protein excretion was increased, and PGE_2 and $PGF_{2\alpha}$ excretion were significantly decreased in these rats (62). TXA_2 inhibitor administration to streptozotocin-induced diabetic rats has also been found to retard hypertensive diabetic nephropathy (56). Thus, the findings of increased urinary TxB_2 in the obese Zucker rat are consistent with previous reports in other diabetic models that linked these changes to renal damage.

In summary, the present study observed changes in CYP450 and COX protein expression primarily in the renal microvasculature of obese Zucker rats at a prehyperglycemic and prehypertensive stage. Renal microvessel CYP4A hydroxylase and COX-1 and COX-2 protein expression were increased in the obese Zucker rat. In contrast, the CYP2C11 and CYP2C23 renal microvessel protein expression was decreased in the obese Zucker rat. The changes in COX protein expression were associated with increases in TXB_2 and decreases in PGE_2 urinary excretion rates. Finally, albumin excretion was apparent in the obese Zucker rat at this prehyperglycemic and prehypertensive stage. Therefore, during the development of obesity-related diabetes, alterations in CYP450 and COX-2 protein expression and the resultant changes in arachidonic acid metabolism could contribute to the renal damage or compensatory mechanisms associated with the disease.

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